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# Leptin inhibits stress-induced apoptosis of T lymphocytes

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## SUMMARY

Leptin, which is secreted by adipocytes, the placenta and the stomach, not only controls appetite through leptin receptors in the hypothalamus but also regulates cell-mediated immunity. In this study we have demonstrated that continuous injection of leptin prevents the reduction in lymphocyte numbers normally observed in fasted and steroid-injected mice. Consistent with leptin-induced protection, we observed up-regulation of the bcl-xL gene as a result of signal transduction via leptin receptors on lymphocytes. We suggest that leptin might contribute to the recovery of immune suppression in malnourished mice by inhibiting lymphocyte apoptosis.

**Keywords** apoptosis bcl-xL corticosteroid leptin

## INTRODUCTION

Nutritional status and immune function are closely related [1–3]. Food deprivation leads to impaired immune responses and an increase in the incidence of infectious disease, although the mechanism by which this occurs has yet to be elucidated. Adipose tissue preserves energy homeostasis through the storage of triglycerides. However, it has been found recently that a number of cytokine-like molecules, such as leptin [4], tumour necrosis factor-$\alpha$ (TNF-$\alpha$) [5] and plasminogen activator inhibitor-1 (PAI-1) [6] are secreted from adipocytes, suggesting that adipose tissue may also play a role in the regulation of the immune and haematopoietic systems.

Leptin is secreted specifically by adipocytes [4], and serum leptin levels are proportional to body mass index. However, the placenta [7] and stomach [8] provide additional sources of leptin. Leptin decreases food intake, increases energy expenditure and reduces body weight via leptin receptors within the ventromedial hypothalamus [9], where leptin functions to inhibit the production of neuropeptide Y which stimulates food intake [10]. The murine leptin and leptin-receptor mutants *ob/ob* and *db/db*, respectively, serve as animal models of obesity, and develop marked obesity and diabetes due to deficiencies in leptin signalling [11]. In contrast, leptin transgenic mice with elevated plasma leptin concentrations lack brown or white adipose tissue, show reduced food intake, and are markedly lean in comparison with non-transgenic littermates [12].

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The leptin receptor is expressed in peripheral tissues such as the kidney, lung and adrenal gland [13,14], and several *in vitro* studies have demonstrated that leptin acts directly on the leptin receptor [15,16]. There are at least five splice variants of the leptin receptor Ob-Ra–Ob-Re, and one of these five variants, Ob-Rb, possesses a long intracellular domain demonstrating homology with gp130, a subunit of the IL-6 family of cytokine receptors [17]. On the other hand, Ob-Ra, one of the shortest forms of the leptin receptor, lacks the STAT3 activation domain and is not considered essential for signal transduction [18].

Recent studies have revealed that Ob-Rb is expressed in fetal liver haematopoietic precursor cells, bone marrow and peripheral T cells [14,19]. In adult human bone marrow, both CD34 positive and negative cells express leptin receptor. These findings suggest the possibility that leptin not only regulates body weight, but also modulates the immune system. Indeed, leptin increases the proliferation of haematopoietic stem cell populations at the multilineage progenitor level [18], enhances alloproliferative mixed-lymphocyte reactions, and reverses cellular immune function in fasted mice [20]. In addition, leptin might act as a growth factor for both myeloid leukaemic cells [21] and lung cancer cells [22]. In addition, human white blood cell counts are correlated with body mass index and serum leptin levels [23]. Moreover, diminished cell-mediated immunity and decreased lymphocyte counts have been reported in *ob/ob* and *db/db* mice [24,25].

We demonstrate here that leptin receptor messenger RNA is expressed in lymphoid tissue, and that leptin both restores the decrease in lymphocyte numbers normally observed in fasted mice and prevents apoptosis of lymphocytes in steroid-injected mice. Consistent with the observed anti-apoptotic effect of leptin, we observed up-regulation of the bcl-xL gene by leptin. We suggest that leptin may contribute to recovery of immune

suppression in malnourished mice by inhibiting lymphocyte apoptosis.

## MATERIALS AND METHODS

*Mice and reagents*

Female C57BL/6 mice (6 weeks old) and C57BL/Ks db/db (6 weeks old) were purchased from SLC (Kyoto, Japan) and CLEA (Tokyo, Japan), respectively. Leptin transgenic mice were bred in our laboratory [12]. Recombinant mouse leptin was purchased from R&D Systems (Minneapolis, USA). Hydrocortisone phosphate was purchased from Banyu Pharmaceutical Co. (Tokyo, Japan). All monoclonal antibodies (MoAbs), including hamster anti-CD3 MoAb (2C11), antimouse bcl-2 MoAb and rat antimouse bcl-x MoAb, were purchased from Becton-Dickinson (Franklin Lakes, USA). N-acetylsphingosine (C2-ceramide) was purchased from Sigma Aldrich Japan (Tokyo, Japan).

*RT-PCR*

Total RNA was extracted from cells using TRIzol® reagent (GIBCO/BRL, Rockville, USA). Five micrograms of total RNA was reverse-transcribed and PCR was performed using specific Ob-Ra and Ob-Rb primers [13].

*Fasting and steroid injection experiments*

Mice were kept without food for 60 h, with repeated intraperitoneal injection of 0, 1 or 10 $\mu$g/g initial mouse body weight of leptin in a solution of phosphate buffered saline (PBS) every 12 h. In a second experiment, 200 $\mu$g/g mouse body weight of hydrocortisone phosphate was administered intraperitoneally; 10 $\mu$g/g body weight of leptin was injected 2 h before and 4 h after administration of hydrocortisone phosphate. Mice were sacrificed 24 h after hydrocortisone injection.

*Cell culture*

$1 \times 10^5$ cells/ml of T cell hybridoma A3·4C6 [26] were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 $\mu$g/ml streptomycin, 0·25 $\mu$g/ml amphothericin B, 2 mM L-glutamine and 0·05 mM 2-mercaptoethanol, with or without recombinant mouse leptin, under conditions of 37°C and 5% $CO_2$ for 24 h. In the steroid-induced apoptosis experiment, cells were cultured with $10^{-6}$ M hydrocortisone for 24 h. In the ceramide-induced apoptosis experiment, cells were cultured with 10 $\mu$g/ml C2-ceramide for 18 h. In another experiment, cells were cultured for 5 h on 96-well plates (Costar 3590), coated with anti-CD3 antibody (10 g/ml) overnight.

*Assay for DNA fragmentation*

Agarose gel electrophoresis was carried out as previously described [27]. DNA was extracted from $1 \times 10^6$ cells/sample and suspended in 20 $\mu$L of lysis buffer (10 mM EDTA, 50 mM Tris HCl (pH 8·0), 0·5% sodium-N-lauroylsarcosinate) and 1 $\mu$L of 10 mg/ml RNaseA was added. Samples were then incubated at 50°C for 30 min, 1 $\mu$L of 10 mg/ml Proteinase K was added and incubated for 30 min at 50°C. Then, samples were analysed by agarose gel electrophoresis.

*Cell staining*

For cell surface analysis, $1 \times 10^5$ thymocytes were stained with FITC-conjugated anti-CD4 MoAb and PE-conjugated anti-CD8 MoAb and analysed by FACSCalibur® (Becton Dickinson), as described previously [27]. For detection of bcl-2 and bcl-xL gene products, cells were washed and fixed in PBS containing 4% paraformaldehyde for 20 min. Cells were then washed and suspended in staining buffer (1% BSA in PBS with 0·1% sodium azide) with 0·1% saponin, followed by incubation with hamster antimouse-bcl-2 MoAb or rat antimouse bcl-xL MoAb for 30 min on ice. After washing, cells were stained with FITC-conjugated antihamster IgG or FITC-conjugated antirat IgG, respectively, for 30 min on ice. After washing, cells were analysed by FACSCalibur®. In order to detect apoptosis, cells were stained with 50 $\mu$g/ml of propidium iodide after fixation with 70% ethanol for 4 h at 4°C and treatment with 100 $\mu$g/ml of RNaseA. After washing, apoptotic cells were determined as the proportion of hypodiploidal cells.

*Northern blotting*

Total RNA was extracted from A3·4C6, spleen and thymus cells using TRIzol® reagent. Samples of 15 $\mu$g of RNA were applied to each lane. After electrophoresis, RNA was transferred to a nylon membrane and hybridized with a $^{32}$P-labelled mouse bcl-xL specific cDNA probe. Autoradiography was performed for 24 h and analysed using BAS 2000® (Fuji Photo Film, Tokyo, Japan).

## RESULTS

To clarify the involvement of leptin in the immune response, we first confirmed the expression of leptin receptor messenger RNA in lymphoid tissue. Both short and long isoforms of the leptin receptor, Ob-Ra and Ob-Rb, were detected in the spleen, thymus and bone marrow by RT-PCR (Fig. 1).

Next, we investigated the effect of leptin on the number of lymphocytes detected in fasted mice. After 60 h of fasting, decreased numbers of thymocytes and splenocytes were detected (Table 1). In the thymus, the $CD4^+CD8^+$ T cell population decreased dramatically from 85% (control) to 25% in fasted mice (Fig. 2a). On the other hand, the proportion of CD4 and CD8 single-positive T cells did not differ among the spleens of fasted and fed control mice (data not shown). Repeated injection of leptin reduced the observed decrease in lymphocyte numbers within the thymus of fasted mice, particularly that of the $CD4^+CD8^+$ T cell population (Table 1, Fig. 2a). The protective effect of leptin was observed to be dose-dependent (Table 1, Fig. 2a). However, one area that remained unclear was whether leptin exerted a direct effect on lymphocytes, or whether it exerted its effect through an indirect mechanism, for example, by counteracting the endocrinological disturbances associated with fasting.

Fasting is widely known to cause the stress-induced release of several hormones *in vivo* [28]. In particular, a steroid hormone derived from the adrenal glands is suspected to play an important role in fasting-induced lymphopenia because lymphocyte numbers do not decrease in adrenectomized mice, even during fasting [29]. To investigate the effect of leptin on the steroid-induced cell death of lymphocytes, hydrocortisone was intraperitoneally injected (200 $\mu$g/g body weight) into mice, with or without leptin (10 $\mu$g/g body weight). As noted during fasting, steroid injection decreased the number of lymphocytes, particularly $CD4^+CD8^+$T lymphocytes, within the thymus. Injection of leptin inhibited the steroid-induced decrease of thymocytes and splenocyte numbers (Table 1) and reversed the observed decline

in $CD4^+CD8^+$ thymocyte numbers (Fig. 2b). This result is consistent with the observed effect of leptin in fasted mice. Moreover, DNA fragmentation of thymocytes in steroid-injected mice was prevented by leptin administration (Fig. 3). In contrast, when leptin-receptor-defective *db/db* mice were treated with hydrocortisone, the proportion of $CD4^+CD8^+$ T cells within the thymus decreased, as was observed for normal mice, but the change was not reversed by injection of leptin (Fig. 2c). This result indicates that the protective effect of leptin against the decline in lymphocyte numbers is related specifically to the binding of leptin to its receptor. When combined, these results strongly suggest that leptin can reverse the decline in T cell numbers due to fasting by preventing steroid-induced apoptosis of lymphocytes by binding to its receptor.

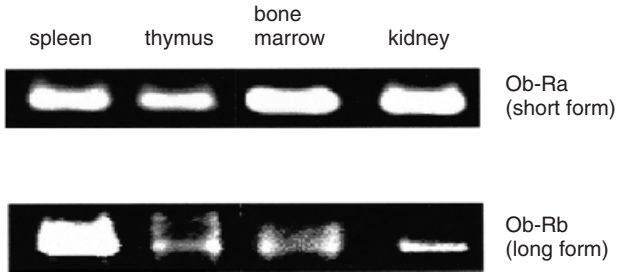


**Fig. 1.** The leptin receptor is expressed in lymphocytes. Short and long isoforms of the leptin receptor, Ob-Ra and Ob-Rb, were detected by RT-PCR in lymphocytes of the spleen, thymus and bone marrow. Kidney served as a positive control for expression of the leptin receptor.

**Table 1.** Leptin recovers the decrease in lymphocytes observed in starved and steroid injected mice

| | Thymus | | Spleen |
|---|---|---|---|
| Treatment | Thymocytes | Double positive thymocytes | Splenocytes ($\times 10^6$) |
| Untreated | 177.0 ± 20.8 | 154.0 ± 25.4 | 125.0 ± 4.80 |
| Starved | 18.2 ± 11.5 | 5.12 ± 5.29 | 15.7 ± 6.15 |
| Starved and leptin treated (1 $\mu$g/g) | 25.8 ± 10.1 | 10.1 ± 6.20 | 28.1 ± 10.3 |
| Starved and leptin treated (10 $\mu$g/g) | 45.2 ± 3.79 | 27.7 ± 4.22 | 50.8 ± 8.29 |
| Untreated | 101.0 ± 6.42 | 83.0 ± 5.26 | 159.0 ± 66.1 |
| Hydrocortisone treated | 12.1 ± 5.25 | 2.39 ± 3.00 | 65.0 ± 16.3 |
| Hydrocortisone and leptin (10 mg/g) treated | 24.1 ± 5.30 | 9.87 ± 5.21 | 102.0 ± 13.1 |

The mice were treated as described in the Materials and methods section. Data are represented as the mean ± s.d., $n = 3$ per group.

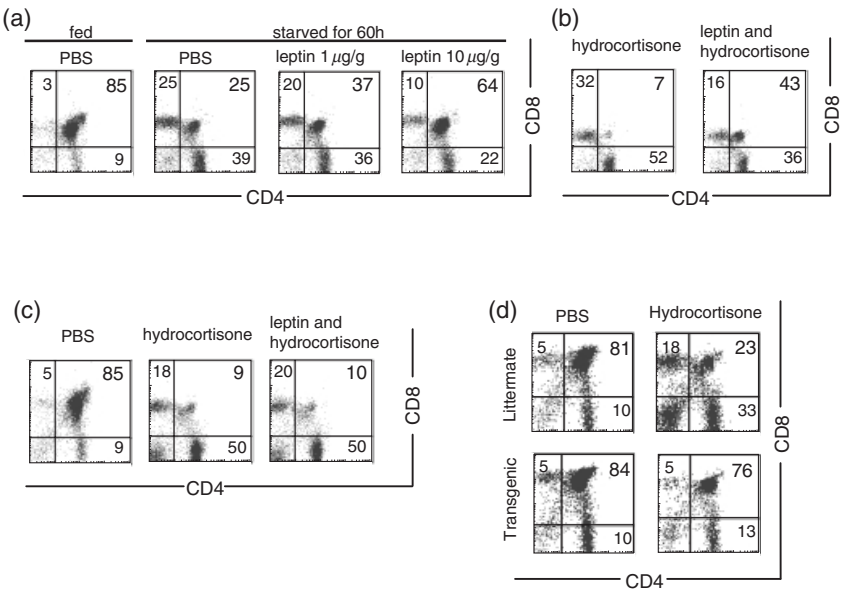


**Fig. 2.** (a) Leptin reverses the decrease in $CD4^+CD8^+$ thymocytes normally observed in fasted mice. (b) Leptin protects against steroid-induced apoptosis of $CD4^+CD8^+$ thymocytes. (c) Leptin cannot rescue thymocytes of *db/db* mice from steroid-induced apoptosis. (d) Thymocytes of leptin transgenic mice are resistant to steroid-induced apoptosis. This figure represents one of three separate experiments in which similar results were obtained. The percentage of fluorescence-positive cells that were detected is indicated in the corresponding squares.

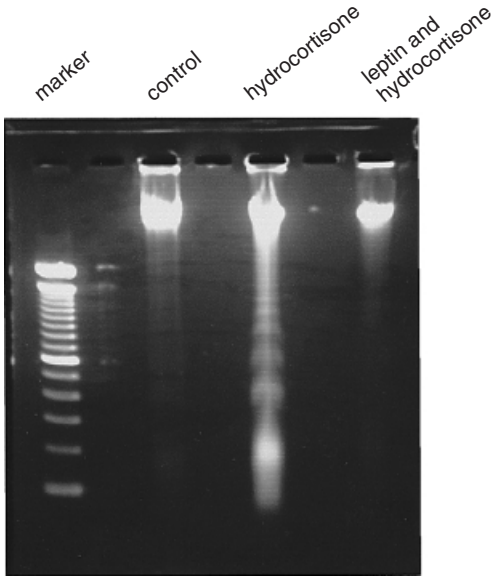


**Fig. 3.** Leptin prevents DNA fragmentation in thymic T cells induced by steroid administration.

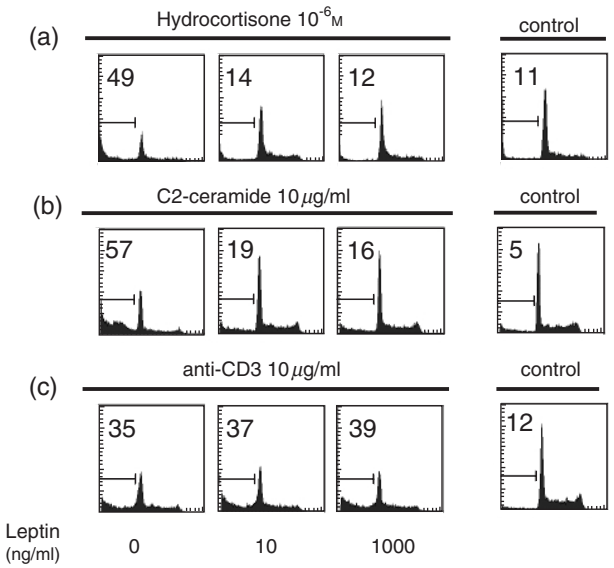


**Fig. 4.** Leptin prevents apoptosis induced by steroid (a) and ceramide (b), but does not prevent that induced by anti-CD3 MoAb (c). The T cell hybridoma, A3·4C6, was preincubated with or without leptin and apoptosis was induced. The proportion of apoptotic cells was analysed by FACSCalibur® and is shown in figures.

Next, we used leptin transgenic mice to confirm the anti-apoptotic effect of leptin on thymocytes. Plasma leptin concentrations in leptin transgenic mice are approximately 12-fold higher than those of non-transgenic littermates [12]. In the present study, no differences in the surface markers of lymphocytes in the spleen, thymus and bone marrow were observed between leptin transgenic mice and non-transgenic littermates (data not shown). However, when hydrocortisone was injected into leptin transgenic mice and non-transgenic littermates, lower levels of $CD4^+CD8^+$ thymocytes were not found in the leptin transgenic mice compared to non-transgenic littermates (Fig. 2d), suggesting that the thymocytes of leptin transgenic mice are protected from steroid-induced apoptosis by maintaining elevated plasma leptin levels.

To clarify the mechanism by which leptin prevents steroid-induced apoptosis, we performed *in vitro* experiments using a murine T cell hybridoma, A3·4C6 [26]. This hybridoma has been shown to be specific for sperm whale myoglobin and to express the leptin receptor (data not shown). After incubation with leptin for 24 h, followed by an additional 24 h with hydrocortisone, apoptotic cells were examined by flow cytometric analysis. Leptin decreased the proportion of apoptotic cells in a dose-dependent manner (Fig. 4a). In contrast, the anti-CD3 antibody-induced apoptotic death of T cells was not prevented by leptin (Fig. 4c), suggesting that leptin is involved in steroid-induced apoptosis but not in activation-induced cell death.

Recent studies have revealed that ceramide is likely to be one of the several second messengers involved in steroid-induced apoptosis [30]. To investigate whether leptin is effective in preventing ceramide-induced cell death, apoptosis of A3·4C6 induced by N-acetylsphingosine (C2-ceramide) was examined. Leptin decreased the number of apoptotic cells induced by ceramide in a dose-dependent manner (Fig. 4b), as it did following steroid-induced apoptosis (Fig. 4a). These results suggest the possibility that leptin prevents steroid-induced apoptosis downstream of ceramide.

As Ob-Rb displays homology with gp130, a common signal transduction molecule, leptin is thought to activate STAT3 in the same way as does gp130 receptor signalling [16]. Recent studies have revealed that STAT3 binds to the promoter region of bcl-xL, an antiapoptotic molecule of the bcl-2 family, and thereby enhances transcription of the protein [31]. We therefore investigated the possibility that the anti-apoptotic activity of leptin can be attributed to increased expression of bcl-xL following activation of STAT3. We performed flow cytometric analysis and Northern blotting to confirm bcl-xL expression at both the protein and messenger RNA level. When the T cell hybridoma A3·4C6, expressing Ob-Rb, was incubated in the presence of leptin for 24 h, expression of cytoplasmic bcl-xL protein was significantly increased in a dose-dependent manner (Fig. 5a). Moreover, bcl-xL messenger RNA in A3·4C6 was enhanced by leptin in a dose-dependent manner (Fig. 5b). Consistent with the results of *in vivo* experiments, bcl-xL messenger RNA was enhanced within the spleen and thymus of leptin-injected mice (Fig. 5c).

## DISCUSSION

The mechanism by which malnutrition leads to immunodeficiency is not fully understood. However, leptin has been reported to be the key link between fasting and immunodeficiency [20], and to inhibit thymocyte apoptosis both *in vivo* and *in vitro* [32]. As fasting increases the production of steroid hormones, it may induce apoptosis of T cells. The present study offers compelling evidence that leptin inhibits the decline in lymphocyte numbers that normally accompanies fasting, in addition to T cell apoptosis following steroid injection. The evidence further suggests that leptin protects lymphocytes through up-regulation of bcl-xL via leptin receptors on lymphocytes. Only one-third of T cell hybridoma cells expressed bcl-xL after leptin stimulation and the observed increase in expression of the bcl-xL gene was not as dra-

matic as that observed following leptin stimulation of fasted mice (Fig. 5). These data suggest that bcl-xL alone is not responsible for the observed inhibitory effect of leptin. Fasting increases the level of steroid hormone within the serum and decreases the amount of circulating leptin [28], both of which can accelerate apoptosis of lymphocytes and impair the immune response.

In contrast to a previous report that found an association between obesity, immune suppression and the presence of infectious disease [33] and cancer [34], our results suggest that obesity might confer resistance to infection. This paradoxical observation can be explained by considering the possibility that the leptin signal is less transducible in obese patients.

Ceramide is generated by hydrolysis of sphingomyelin followed by activation of acidic sphingomyelinase. It functions as an intracellular second messenger, mediating the sphingomyelin signalling pathway. Recently, ceramide has been examined as a common intermediator of several apoptotic stimuli, including steroid administration [30]. However, it is not involved in Fas-induced apoptosis [35,36]. The apoptotic effect of ceramide is due to its induction of cytochrome c release from mitochondria [37]. The bcl-2 gene family can inhibit ceramide-induced apoptosis [38,39].

The present study confirms the findings of reduced numbers of lymphocytes in fasting, and illustrates that leptin prevents the decline in lymphocytes during fasting, in addition to preventing apoptosis of T cells following steroid injection [32]. This may be explained partly by the ability of leptin to up-regulate bcl-xL through the leptin receptors on lymphocytes. However, leptin might not be able to rescue a T cell hybridoma from activation-induced cell death through CD3 stimulation, which is known to involve Fas and the Fas ligand [40–42]. The different effects of leptin on steroid-induced apoptosis and activation-induced cell death might be explained by the bcl-2 related gene products, which prevent apoptosis downstream of ceramide.

Leptin was found originally to modulate body weight but, more recently, it has become recognized as an immune regulator. Administration of leptin to fasted mice has been observed to reverse impairment of T cell function [20]. Although the present study illustrates that leptin inhibits the stress-induced apoptosis of T cells, other effects with regard to immunity remain unclear.

As shown in the present study, lymphocytes can be preserved during fasting by administration of exogenous leptin. This suggests that leptin might be used therapeutically to treat immunodeficiency caused by severe malnutrition and cachexia in cancer and AIDS patients. Such applications may be assessed by further investigation using mouse models of AIDS [43]. On the other hand, it is possible that the anti-apoptotic effects of leptin might result in autoreactive T cells and lead to the development of autoimmune disease. The possibility that leptin might lead to autoimmunity is currently under investigation using our leptin transgenic mice.

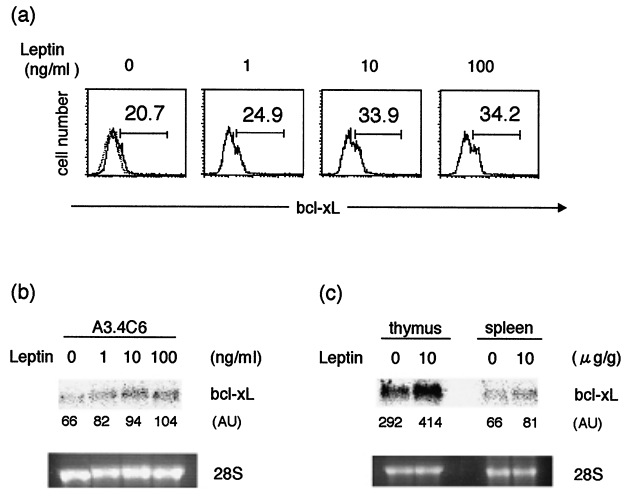


**Fig. 5.** (a) Leptin induced bcl-xL protein in the T cell hybridoma. The T cell hybridoma, A3·4C6 was incubated in the presence or absence of leptin for 24 h. Expression of the bcl-xL gene was detected with cell-staining using antibcl-xL MoAb and a fluorescent conjugated second antibody. The dotted line indicates cell staining using the fluorescent conjugated antibody without antibcl-xL MoAb. (b) Expression of bcl-xL messenger RNA is enhanced by leptin. A3·4C6 were incubated with graded concentrations of leptin for 24 h and RNA was extracted as described in the materials and methods section. (c) C57/BL6 mice were injected with either PBS or leptin (10 $\mu$g/g) and RNA was extracted from the thymus and spleen 24 h later. Northern blotting was performed as described in the materials and methods section. The 28S ribosomal RNA bands were stained with ethidium bromide and are shown in the lower panels of this figure. The relative band intensities of bcl-xL are indicated by assignment of arbitrary units (AU).

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